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Research Article

Gradient patterns in the Tatra Mountain lakes revisited with invertebrate DNA metabarcoding

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Abstract

Monitoring changes in freshwater fauna requires timely, efficient, and comprehensive data collection, needs that are increasingly met by molecular methods. This is especially important in remote regions along environmental gradients, such as the Tatra Mountain lakes in Slovakia. In this study, DNA metabarcoding was applied to littoral benthic invertebrates collected from lakes in three valleys, spanning a gradient from the subalpine to alpine zones. Ordination analyses grouped the lakes based on community composition, and these groups were evaluated in relation to altitude, physico-chemical properties, indicator species, and previous data.

To assess their additional value, surface sediment samples from the littoral zone were also analyzed using metabarcoding. The results revealed two distinct lake groups, primarily differentiated by altitude, alkalinity, and other correlated environmental parameters. The placement of specific lakes within these groups corresponded with known patterns of recovery from acidification, catchment characteristics, and potentially climate change or lake connectivity.

Metabarcoding-based indicator species analysis successfully identified even taxa that had been previously overlooked or misidentified in morphology-based studies. While sediment metabarcoding provided limited supplementary information, its ordination patterns aligned with those from invertebrate analyses.

Overall, our study of an alpine lake system supports broader trends: species richness declines with increasing elevation; community composition reflects environmental gradients; and DNA metabarcoding offers high taxonomic resolution and valuable insights into freshwater invertebrate biodiversity.

Key words: Alpine indicators, amplicon sequencing, aquatic fauna, biomonitoring, genetic diversity, water composition

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Introduction

Benthic invertebrates are an essential component of freshwater ecosystems, where their application in bioindication and biomonitoring has a long-established tradition (Birk et al. 2012). The efficiency of biodiversity surveys has recently

been enhanced by the DNA metabarcoding approach (Hebert et al. 2003; Deiner et al. 2017; Leese et al. 2018; Pereira-da-Conceicoa et al. 2021). Advances in molecular methods have facilitated research into the response of invertebrates to gradual changes in environmental conditions (Cordier et al. 2018; Zizka et al. 2020; Beermann et al. 2021; Madge Pimentel et al. 2024). It is evident that among the natural systems that provide such environmental gradients are the plentiful glacial lakes located along mountain valleys. These lakes have experienced a similar evolution in macroclimatic conditions and acid deposition, yet exhibit variations in microclimatic and nutrient conditions, which are associated with disparities in land cover and vegetation zones across elevational gradients (Catalan et al. 2009; Čiamporová-Zaťovičová et al. 2010; Novikmec et al. 2013; Kopáček et al. 2024). Invertebrate communities in such remote 'aquatic islands' are frequently isolated, and possess distinct features (Hamerlík et al. 2014; Bitušík et al. 2017; Čiamporová-Zaťovičová and Čiampor 2017). However, they are not protected from widespread anthropogenic threats (Sánchez-Bayo and Wyckhuys 2019; Albert et al. 2021; Priya et al. 2023) and have long been considered sensitive to climate change and air pollution, which compound the already harsh alpine conditions (Mosello et al. 1999; Hořická et al. 2006; Fjellheim et al. 2009; Stuchlík et al. 2017).

Until now, assessments of benthic fauna in the littoral zone of alpine lakes across environmental gradients have been based on morphology rather than DNA metabarcoding (Krno et al. 2006; Fjellheim et al. 2009; Čiamporová-Zaťovičová et al. 2010). The pilot metabarcoding study demonstrated a clear distinction between lakes located in the forest zone of the Tatra Mountains and those in the (sub)alpine zone (Vargovčík et al. 2024). In addition to altitude, decades of research in this small alpine system have described specific characteristics of lakes (and their catchments) that influence the varying acidification responses and recovery of lake biota across various taxa (Hořická et al. 2006; Krno 2006; Bitušík et al. 2010; Bitušík et al. 2017; Stuchlík et al. 2017; Kopáček et al. 2024). Conversely, the capacity to discern all organisms present at a given site is invariably constrained by the limitations inherent in the methodologies employed for sampling and processing. In the context of metabarcoding, this limitation is further compounded by the specificity of PCR primers (Deiner et al. 2017; Leese et al. 2018; Pereira-da-Conceicoa et al. 2021). Metabarcoding studies examining the effects of altitude have typically focused on other communities, such as plankton (Pritsch et al. 2023; Salmaso et al. 2024), biofilms (Salmaso et al. 2024), or sedimentary bacteria and microeukaryotes, with macroinvertebrates only appearing as bycatch (Pearman et al. 2020).

Excluding mountain lakes, the metabarcoding approach has been employed to validate a gradient of greater diversity towards lower altitudes for stream fish communities (Bylemans et al. 2018), terrestrial insects (Måsviken et al. 2023), and soil fungi (Blaschke et al. 2023). Multivariate analyses of metabarcoding data have successfully revealed altitudinal trends in community composition, even when these were not evident from diversity indices alone (Dopheide et al. 2019; Littlefair et al. 2019; Stewart et al. 2021).

A substantial body of research has demonstrated the compatibility of parallel identification through conventional methods alongside metabarcoding, and the great potential of the DNA metabarcoding approach (Elbrecht et al. 2017; Martins et al. 2019; Leese et al. 2021; Pereira-da-Conceicoa et al. 2021;

Blaschke et al. 2023; Måsviken et al. 2023). Consequently, as opposed to allocating resources to further comparisons of benthic metabarcoding and morphological identification, it was decided to enhance the analyses with matched samples of surface sediment. In addition to concentrating shed and settled material, including environmental DNA (Turner et al. 2015), sediments serve as natural habitats for diverse living organisms that might otherwise be overlooked (Fjellheim et al. 2009; Pearman et al. 2020; Pawlowski et al. 2024).

This study builds upon previous research on mountain lake invertebrates by employing DNA metabarcoding to assess gradual variations from the subalpine to alpine zone within the High Tatras, the highest region of the Carpathians. The hypothesis was formulated that at least two distinct lake categories would emerge from community dissimilarities observed via DNA metabarcoding of littoral benthic invertebrate samples, representing a gradient of altitude and lake environmental parameters. In addition, the indicator species analysis was anticipated to ascertain species that have been previously identified as indicative of subalpine versus alpine conditions. Furthermore, the potential for conducting a similar analysis on surface sediment samples was assessed.

Methods

Study area

The Tatra Mountains—the highest part of the Carpathian chain, reaching up to 2,655 m a.s.l.—lie along the border between Slovakia and Poland and form a watershed between the Baltic and Black Seas (Fig. 1). Together with smaller or seasonal lakes, there are 260 lakes in the Tatra Mountains (Gregor and Pacl 2005; Kopáček et al. 2015). These lakes are almost exclusively of glacial origin, mostly located in the alpine zone above 1,800 m a.s.l. In recent decades, the physico-chemical properties and the reaction to acidic deposition in the Tatra lakes have been thoroughly investigated (Kopáček et al. 2019; Kopáček et al. 2024).

In this study, we selected 17 lakes located in four gradient series. When selecting these series, we focused on valleys with a chain of at least three (sub)alpine permanent lakes distanced by > 50 m of altitude (Table 1, Fig. 1). Three such valleys were selected: Bielovodská, Veľká Studená, Mlynická. The fourth series is a triplet of lakes considered suitable for gradient studies due to their similar hydromorphology and altitudinal differences of ~215 m, although located in different but nearby valleys (Hamerlík and Bitušík 2009; Čiamporová-Zaťovičová et al. 2010). Two exceptional lakes were included: Starolesnianske pleso in the Veľká Studená Valley severely affected by acidification in 1980s (Svitok et al. 2021), and Štrbské pleso (Mlynická Valley), which is exposed to intense human activities.

Granodiorite bedrock and acidic soils prevail in the catchment area of the selected lakes. The catchments have varying proportions of scree fields—containing sparse till soils beneath surface stones—and alpine meadows growing on shallow, undeveloped podzols, leptosols and regosols (Kopáček et al. 2015). Physiographic parameters of the lakes and their catchments are summarized in Suppl. material 1: table S1. Recent data on physico-chemical properties of the lake water originates from Kopáček et al. (2024) and unpublished data (Suppl. material 1: table S2). Details on the underlying analytical methods were described by Kopáček et al. (2015, 2024).

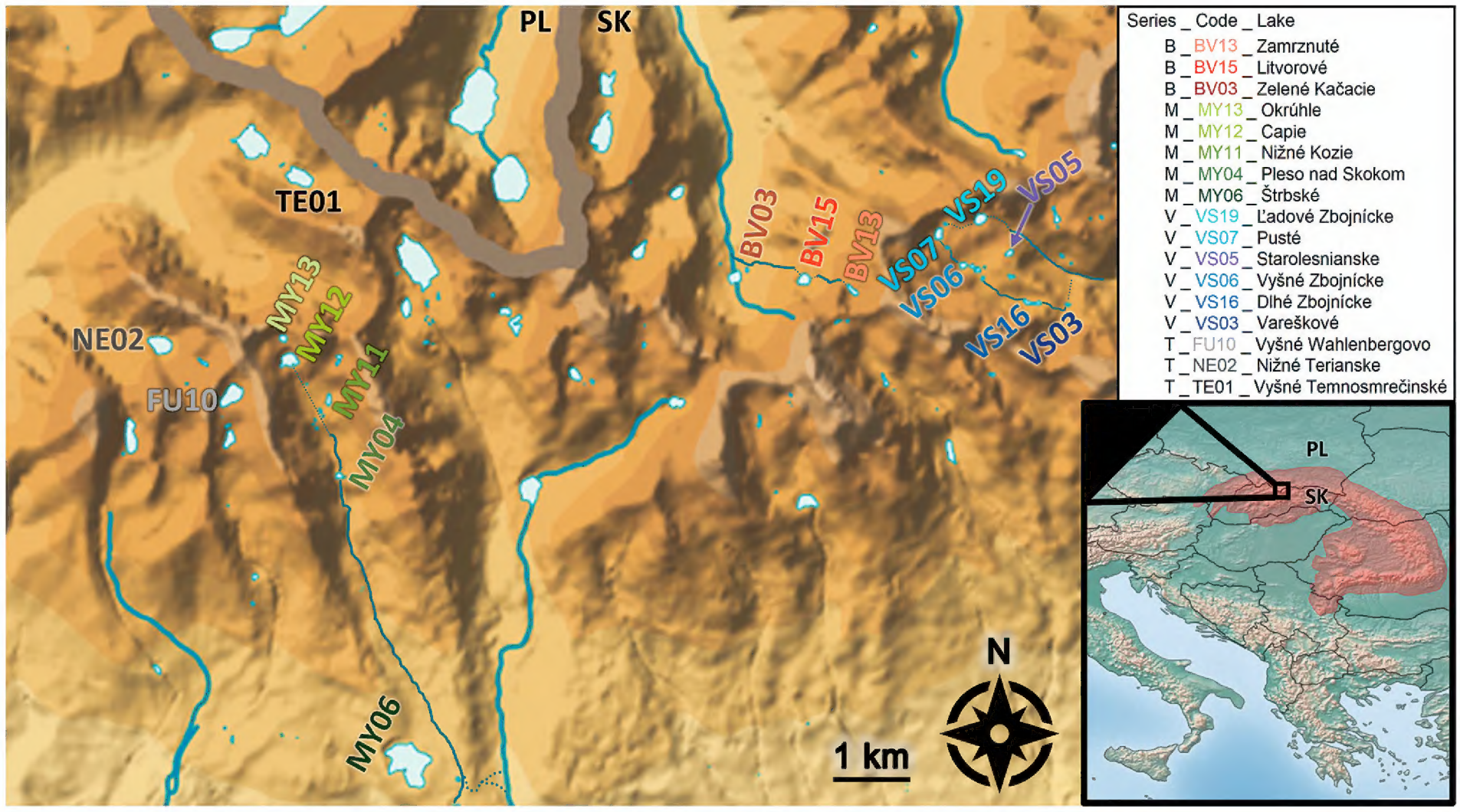

Figure 1. Study sites in the selected gradient series of the Tatra Mountain valleys. Broader European background is in the bottom right, with the Carpathian chain highlighted. Made with QGIS 2.18 qgis.org and modified from figure by SANtosito (2021) distributed under CC BY-SA 4.0 (the same license applies to this Fig. 1).

Sampling and laboratory procedures

The fieldwork was carried out in early September 2017–2022. Each lake was sampled in at least two years (Table 1) to minimize the sampling error and to prevent misrepresentation of the lake communities due to possible fluctuations between years. Altogether, 42 lake visits led to 336 sample replicates analyzed, following the sampling and replication scheme described below. At each visit, single bulk sample of benthos was collected using kicking technique (Frost et al. 1971) with net of 0.3 mm mesh size, at depths approximately up to 1 m. The material was gathered from all accessible types of littoral habitats and substrates in 10 minutes of sampling effort. It was then decanted and carefully deprived of large plant fragments if present. The bulk sample was stored in a 1 L bottle and fixed with 96% ethanol, added to twice the height of sample in the bottle. In parallel, two samples of ~25 mL surface sediment were collected from shore into 50 mL falcon tubes attached to a 1–3 m pole. Upon removing excess water, the tubes were filled to the top with 96% ethanol. Samples were stored at -20 °C.

DNA processing and sequencing was performed yearly in batches. Anti-contamination measures included sterile and protective equipment, work in hoods and spaces dedicated to DNA metabarcoding, and multiple negative controls: 6 at DNA extraction, 41 at amplification, and 75 indexing controls. Animal material was thoroughly picked from each bulk sample, dried at room temperature, and homogenized for 9 min at 6,000 RPM and reverse every 50 s in a ball milling tube BMT-20-S for Ultra-Turrax Tube Drive (IKA, Staufen, Germany). The homogenate was transferred into 1.5 mL tubes in two replicates of ~25 mg and the DNA was isolated with DNeasy Blood & Tissue Kit, following the manufacturer's protocol (Qiagen, Hilden, Germany).

Table 1. List of sampling sites, ordered by gradient series and altitude (Gregor and Pacl 2005). Column Years indicates when the samples were taken for the purposes of this study. For other lake parameters see Suppl. material 1: table S1.

Code	Lake name ("pleso")	Valley ("dolina")	Series	Altitude [m]	Years	Coordinates
BV13	Zamrznuté p.	Bielovodská d.	B	2040	2021, 2022	49.1764°N, 20.1379°E
BV15	Litvorové p.	Bielovodská d.	B	1860	2021, 2022	49.1772°N, 20.1288°E
BV03	Zelené Kačacie p.	Bielovodská d.	B	1575	2021, 2022	49.1782°N, 20.1177°E
MY13	Okrúhle p.	Mlynická d.	M	2105	2017, 2021, 2022	49.1702°N, 20.0353°E
MY12	Capie p.	Mlynická d.	M	2075	2021, 2022	49.1679°N, 20.0355°E
MY11	Nižné Kozie p.	Mlynická d.	M	1942	2021, 2022	49.1638°N, 20.0435°E
MY04	Pleso nad Skokom	Mlynická d.	M	1801	2021, 2022	49.1547°N, 20.0450°E
MY06	Štrbské p.	Mlynická d.	M	1346	2019, 2022	49.1225°N, 20.0608°E
VS19	Ľadové Zbojnícke p.	Veľká Studená d.	V	2057	2019, 2022	49.1834°N, 20.1608°E
VS07	Pusté p.	Veľká Studená d.	V	2056	2021, 2022	49.1817°N, 20.1536°E
VS05	Starolesnianske p.	Veľká Studená d.	V	1988	2019, 2022	49.1804°N, 20.1670°E
VS06	Vyšné Zbojnícke p.	Veľká Studená d.	V	1962	2021, 2022	49.1786°N, 20.1586°E
VS16	Dlhé Zbojnícke p.	Veľká Studená d.	V	1894	2020, 2021, 2022	49.1744°N, 20.1722°E
VS03	Vareškové p.	Veľká Studená d.	V	1834	2020, 2021, 2022	49.1743°N, 20.1771°E
FU10	Vyšné Wahlenbergovo p.	Furkotská d.	T	2157	2019, 2020, 2021, 2022	49.1635°N, 20.0270°E
NE02	Nižné Terianske p.	Nefcerka	T	1940	2020, 2021, 2022	49.1702°N, 20.0110°E
TE01	Vyšné Temnosmrečinské p.	Temnosmrečinská d.	T	1725	2017, 2020, 2021, 2022	49.1896°N, 20.0368°E

The sediment samples were dried in single-use plastic containers at 40 °C to remove ethanol, returned to the falcon tubes, filled with fresh phosphate buffer (0.735 g Na_2HPO_4, 0.099 g NaH_2PO_4 in 50 mL of distilled water per sample, Taberlet et al. 2012), and mixed for 30 minutes in Multi Bio RS-24 rotator (Biosan, Riga, Latvia). After centrifugation at 4,000 RCF for 20 minutes, the DNA in the supernatant was collected with a cellulose acetate filter (0.45 µm pores, Sartorius, Göttingen, Germany) and then isolated with the DNeasy Blood & Tissue Kit (Qiagen).

Amplicon sequencing libraries were prepared as in Vargovčík et al. (2024) and details are provided in Suppl. material 2. Briefly, the two-step PCR using BF3/BR2 primers for a 418 bp fragment of the standard cytochrome oxidase subunit I (COI) barcoding region was performed in duplicates for each DNA extract (Hebert et al. 2003; Elbrecht and Steinke 2019; Elbrecht et al. 2019). The PCR products were pooled, purified and sequenced with MiSeq, Reagent Kit v3 for 2 × 300 bp (Illumina, San Diego, USA). Sequencing took place either at the Comenius University Science Park, or at the Institute of Chemistry, Slovak Academy of Sciences.

Data analysis

Data analyses were conducted in R software v4.3.1 (R Core Team 2016; Wickham et al. 2019). Packages corrplot v0.92 (Wei and Simko 2021) and Hmisc v5-1.0 (Harrell Jr 2023) were employed to inspect Pearson correlations among all environmental parameters. Due to the presence of strong correlations, we identified six representative physico-chemical parameters: TP = total P, TN = total N, Ca, Si, SO_4, temperature at the time of sampling. The selection process was based on iterative removal of parameters with the highest variance inflation factor (VIF) until all remaining parameters achieved VIF < 5 (package car v3.1-2, Fox and Weisberg 2019). The parameter NH_4-N was then removed manually, because its values were close to or below the detection limit.

Preliminary visualization of all the physico-chemical data was achieved with the package vegan v2.6-4 (Oksanen et al. 2022). Specifically, the Euclidean distance matrix calculated from all values in Suppl. material 1: table S2 was subjected to Principal Coordinates Analysis (PCoA) with the cmdscale function. For the sake of readability, only the six representative parameters were displayed by passive projection into the PCoA. For the same reason, the ordination diagrams in this study show ellipses of standard deviation to present the degree of variability among individual data points per lake. Complementary physiographic parameters were passively projected into the ordinations and evaluated based on their coefficient of determination (R^2).

The DNA sequencing data was processed as in Vargovčík et al. (2024) with slight modifications. In short, the reads were demultiplexed, primers trimmed (Martin 2011), paired ends merged (Zhang et al. 2013), and filtered for maximum 1 expected error before denoising, filtering by length (Rognes et al. 2016) (418 ± 15 bp) and compliance with codon table (Andújar et al. 2021), chimera removal and OTU clustering by 97% identity (Rognes et al. 2016). The OTUs (operational taxonomic units) were also curated in terms of co-occurrence (Frøslev et al. 2017), ≥ 85% sequence similarity to reference from invertebrate taxa (Ratnasingham and Hebert 2007; Buchner and Leese 2020), negative control subtraction, read abundance ≥ 0.01% per replicate, and consistency of occurrence (details in Suppl. material 2). After reviewing the taxon list, only OTUs of aquatic invertebrates were retained.

Rarefaction curves were produced with the R package vegan, function rarecurve. They served for evaluation if the samples had sufficient read coverage to reach an asymptote for OTU richness in the step before removing rare occurrences. By this point, it was evident that the data from sediments was affected by the high content of non-invertebrate sequences. Therefore, further evaluation was based on the primary bulk-sample dataset, while certain complementary analyses were performed for the sediment data, as described below.

To evaluate the patterns of grouping among lake communities, Jaccard dissimilarities were calculated for the presence-absence dataset from bulk metabarcoding. Hierarchical clustering analysis was then applied on the dissimilarity matrix. The optimal number of clusters was tested using the indexes and methods available in the package NbClust v3.0.1 (Charrad et al. 2014). The clustering outcomes are presented here using the package factoextra v1.0.7 (Kassambara and Mundt 2020). The complete linkage method was selected due to the high value of Pearson's coefficient of correlation (0.811) between the community distance matrix and cophenetic distances in the dendrogram.

The dissimilarity matrix from the bulk dataset was also modeled with the six representative parameters, using the capscale function for distance-based redundancy analysis (dbRDA). The six constraints in dbRDA were the parameters that passed the abovementioned selection process with VIF < 5. Before dbRDA, values from the physico-chemical measurements were averaged per lake. To assess the relationship between the patterns of lake community composition and environmental parameters, we compared the outcomes of dbRDA and hierarchical clustering. Linear mixed model was used to test the differences in OTU richness between the two major lake groups identified by clustering (package glmmTMB v1.1.7, Brooks et al. 2017). In more detail, Wald Z statistics and two-tailed tests were employed to evaluate *p* values. The dependent variable in the

model was OTU richness. The independent variable of interest was the factor with two levels representing the high- and low-altitude lake groups. Another independent variable was log-transformed read count to control for its potential effect on OTU richness. Nested random effect terms for lakes within gradient series were included as well. Gaussian distribution family was used, because the values of OTU richness had to be transformed by square-root to satisfy model assumptions (checked with DHARMa v0.4.6, Hartig 2022). Marginal and conditional R^2 values were calculated using MuMIn v1.47.5 (Bartoń 2023).

Potential indicator OTUs for the respective groups of lakes were highlighted via the fidelity and specificity metrics obtained with the function multipatt from indicspecies v1.7.14 (De Cáceres and Legendre 2009). To retain only cases of consistent indication across valleys, the indicspecies outcomes were checked against the OTU occurrence dataset, especially attending to the OTUs highlighted by R summary, OTUs with IndVal > 0.5 for one lake group but < 0.5 for the other group. Indicator potential is reported even for species with multiple OTUs, also checking their BIN assignments (barcode index number from the BOLD database).

Analysis of variance (ANOVA) was then used to assess patterns of gradient study design in the bulk metabarcoding dataset. In more detail, the variability in fauna composition was attributed to the valley and lake levels based on the variance components from hierarchical ANOVA of ellipse areas in the dbRDA diagram. Due to possible inconsistencies between the variation in ellipse areas and the original variation in multidimensional dataset, the same purpose was pursued also by another approach: modelling of the dissimilarity matrix in partial dbRDA with lake term as the constraint and gradient series as condition.

The dbRDA, ANOVA and partial dbRDA were repeated separately even for the sediment metabarcoding dataset. Mantel test and coinertia analysis (package ade4 v1.7-22, Dray and Dufour 2007) were used to evaluate the similarity between the Jaccard distance matrices from bulk and sediment metabarcoding.

Results

The initial number of demultiplexed sequencing reads obtained after primer trimming was 12,776,087. After processing and curation, the dataset contained 5,693,319 reads (mean 31,834 ± 17,663 SD in bulk samples; 2,054 ± 4,636 in sediment) clustered in 411 aquatic invertebrate OTUs (35 ± 13 in bulk; 6 ± 5 in sediment). Sufficiency of read coverage for all bulk samples was shown by their long rarefaction plateaus and similar OTU richness among replicates (Suppl. material 3: fig. S1). This was not common for the sediment samples, where the majority of sequences were not identified as invertebrates (Suppl. material 3: fig. S1).

Already in the initial data, the sum of reads from bulk samples was 59% higher than from sediment samples. During sequence processing, the pre-clustering steps removed 18% of reads from bulk samples vs. 36% from sediment samples. Further curation with taxonomic filtering discarded 5% vs. 87% reads, representing 55% vs. 96% OTUs from the respective sample types (Suppl. material 3: fig. S1).

The final bulk dataset contained 343 OTUs and comprised to a great extent of Diptera (141 OTUs, 32% reads), followed by EPT orders (Ephemeroptera, Plecoptera, Trichoptera) and annelids (Fig. 2). Other arthropods, rotifers, mollusks and a planarian comprised smaller fractions of data. Except for three clitellates and a tardigrade, all OTUs contained at least order-level taxonomic information.

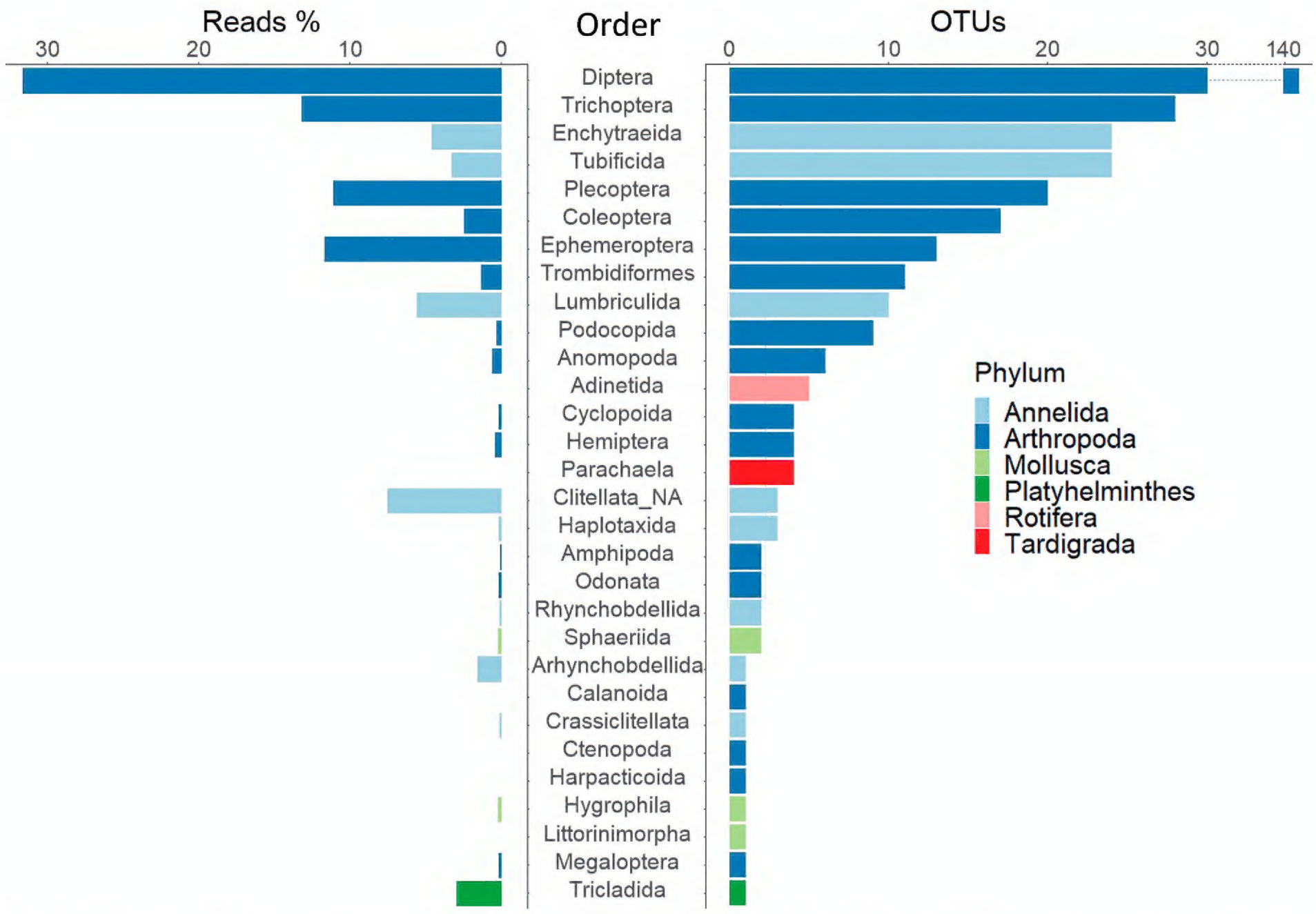

Figure 2. Aquatic OTU counts and read proportions for invertebrate orders detected with bulk metabarcoding. The omitted range of 30 - 140 OTUs is marked by dashed lines.

The proportion of assigned OTUs dropped to 94%, 86%, 72% at family, genus and species levels, respectively. The curated sediment dataset had 176 OTUs total, with 81%, 78%, and 65% assigned to family, genus, and species, respectively. Crustacean reads prevailed in the curated dataset for sediment samples, which differed from bulk even by capturing the phylum Gastrotricha (Suppl. material 3: fig. S2).

Clustering of the bulk metabarcoding dataset revealed that the replicates per lake visit were highly similar (Suppl. material 3: fig. S3) and 25% of tests recommended 20 or more clusters. However, most tests suggested that fewer than 7 clusters would be optimal, as samples from multiple visits per lake often clustered together. Lake Štrbské MY06 emerged as a clear outlier from the tests across multiple agglomeration methods and two major groups of lakes could be identified as well (Suppl. material 3: fig. S3). Several tests suggested that the lake Starolesnianske VS05 is another outlier.

One of the identified lake groups consisted of high-altitude lakes BV13, BV15, MY13, MY12, MY11, VS19, FU10, and NE02. The other group included the remaining lakes and is referred to as “low-altitude group”, although VS06 and VS07 lie above 1950 m a.s.l. The two groups differ in richness by -1.588 OTUs on square-root scale, with standard error SE = 0.305 and statistical significance $p < 0.001$. The intercept was 6.842 ± 1.022 SE and the effect of log-transformed read count was marginal (-0.178 ± 0.097 SE, $p = 0.067$). Marginal and conditional R^2 of the model was 0.495 and 0.728, respectively.

The same two groups of lakes formed along the first axis of dbRDA from bulk dataset (Fig. 3). The second dbRDA axis showed the dissimilarity of the two outlier lakes Štrbské and Starolesnianske. The constrained variance represented 27.3% of the total variance. The variance explained by the first and second axis represented 33.4% and 22.1% of the constrained variance, respectively. The axis separating the two lake groups was related to the concentrations of Ca, SO_4, and altitude (Fig. 3). These parameters were also correlated with pH, conductivity, alkalinity, etc. (Suppl. material 3: fig. S4). The segregation of outlier lakes was reflected by TP, TN, Si, temperature, and catchment cover (soil vs. scree or bedrock), which were correlated with dissolved organic carbon (DOC), total organic nitrogen (TON), and NO_3-N.

Concerning possible indicator OTUs, the most notable was the well-defined occurrence of two OTUs, assigned to *Tubifex tubifex* BOLD:ACA9222 and *Micropsectra notescens* BOLD:AAC4098. They were detected only in the low-altitude lake group (100% specificity) and in all of those lakes (100% lake fidelity, Suppl. material 1: table S3). No other OTU achieved full lake fidelity and only twelve other OTUs manifested full specificity towards their respective group. On the more detailed level of replicates, not even the two abovementioned OTUs had full specificity.

The absolute number of the recognized indicator OTUs (35 total) was more than twice larger for low-altitude than high-altitude lake group, but they had similar proportions in terms of broader taxonomic units: roughly half belonged to chironomids, a sixth to clitellates and the rest were stoneflies, seed shrimps, or water mites, a caddisfly, a beetle and a bivalve (Suppl. material 1: table S3). Identical species assignment appeared for two pairs of indicator OTU candidates, identified as *Heterotrissocladius marcidus* and *Pseudodiamesa nivosa*. Overall, 28 species in the bulk dataset contained more than one OTU (70 OTUs total), sometimes even splitting into different BINs. These closely related OTUs showed co-occurrence rather than conflicts in indicator potential (Suppl. material 1: table S3).

The complementary ordination diagrams from PCoA of physico-chemical measurements and from dbRDA of sediment dataset were similar to the bulk metabarcoding dbRDA (Suppl. material 3: figs S5, S6, Fig. 3). Again, the two outlier lakes MY06 and VS05 were segregated from the remaining lakes, which were roughly ordered by altitude, Ca, SO_4, etc. However, the groups of high- and low-altitude lakes were not clearly separated in Suppl. material 3: figs S5, S6. The ellipses in sediment dbRDA overlapped to a high degree, in contrast to the more compact ellipses in bulk dbRDA (Suppl. material 3: fig. S6, Fig. 3). The Mantel test and coinertia analysis implied nonrandom similarities between bulk and sediment datasets, regarding the placement of corresponding replicates in the multivariate space (Fig. 4).

Tracking the variability in the bulk metabarcoding dataset via partial dbRDA showed that less inertia was attributed to the valley level (14%) than to the lake level (45%). The variance in ellipse area followed a similar trend (23% and 66%, respectively). Unlike partial dbRDA, the ellipse-area approach was strongly affected by the two outlier lakes, whose exclusion would change the outcomes to > 99.99% variance at the lake level. Similar patterns were observed in the sediment dataset: variability on the level of valleys was lower (8% in partial dbRDA, 7% in the ellipse areas) than on the lake level (23%, 84%, respectively).

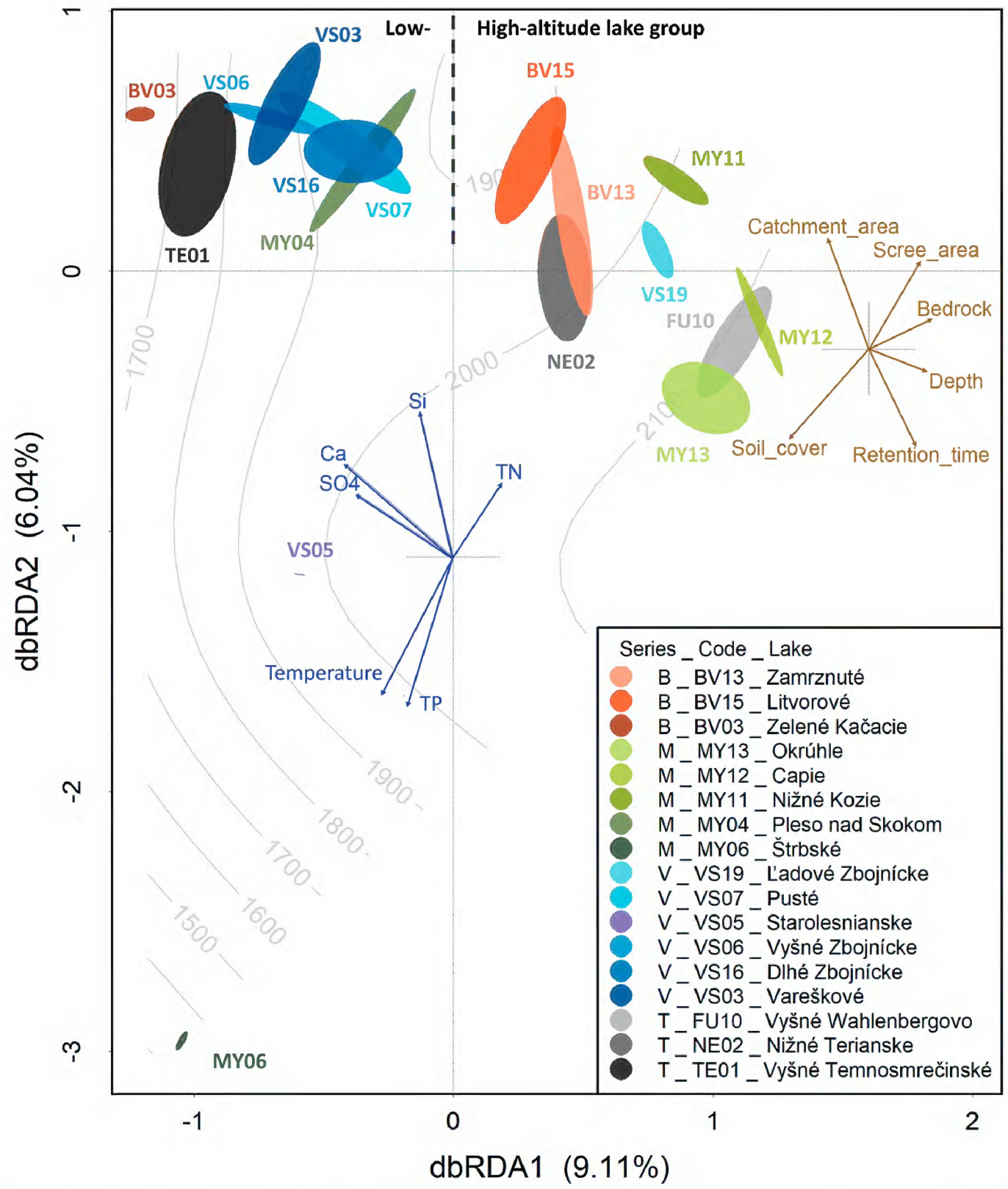

Figure 3. Distance-based redundancy analysis for the final bulk metabarcoding dataset. Ellipses of standard deviation are shown, summarizing replicates from each lake (colors correspond to the gradient series and altitude). Axis labels contain the respective proportions of variance explained from the total variance. Constraints in the analysis were the selected environmental parameters (blue vectors). Altitude (contour lines) and other physiographic parameters were projected passively (brown vectors, displayed if $p < 0.05$). Vector size was reduced 0.7 times and their positions adjusted for clarity. Note that other parameters were highly correlated with TP (especially DOC, TON, NO_3-N) and Ca (pH, alkalinity). Catchment parameters of the lake Štrbské were not included, as its catchment is not well defined. TN = total N, TP = total P, DOC = dissolved organic carbon, TON = total organic N. The low- and high-altitude lake groups identified by hierarchical clustering are distinguished by the dashed line.

Discussion

The lake series and methods selected in this study have proven suitable for capturing the altitudinal gradient, even though the analyzed lakes display non-uniform hydromorphology. This conclusion is supported by trends in physico-chemical properties (Suppl. material 3: fig. S5) and by the observed variability in community composition, which was greater among lakes than among valleys. Although such variability is not excluded even in a typical hierarchical spatial design (Underwood and Chapman 1998), our findings align with the assumption that altitude is a stronger structuring factor than geographic proximity for our study sites (Lepš and Šmilauer 2020).

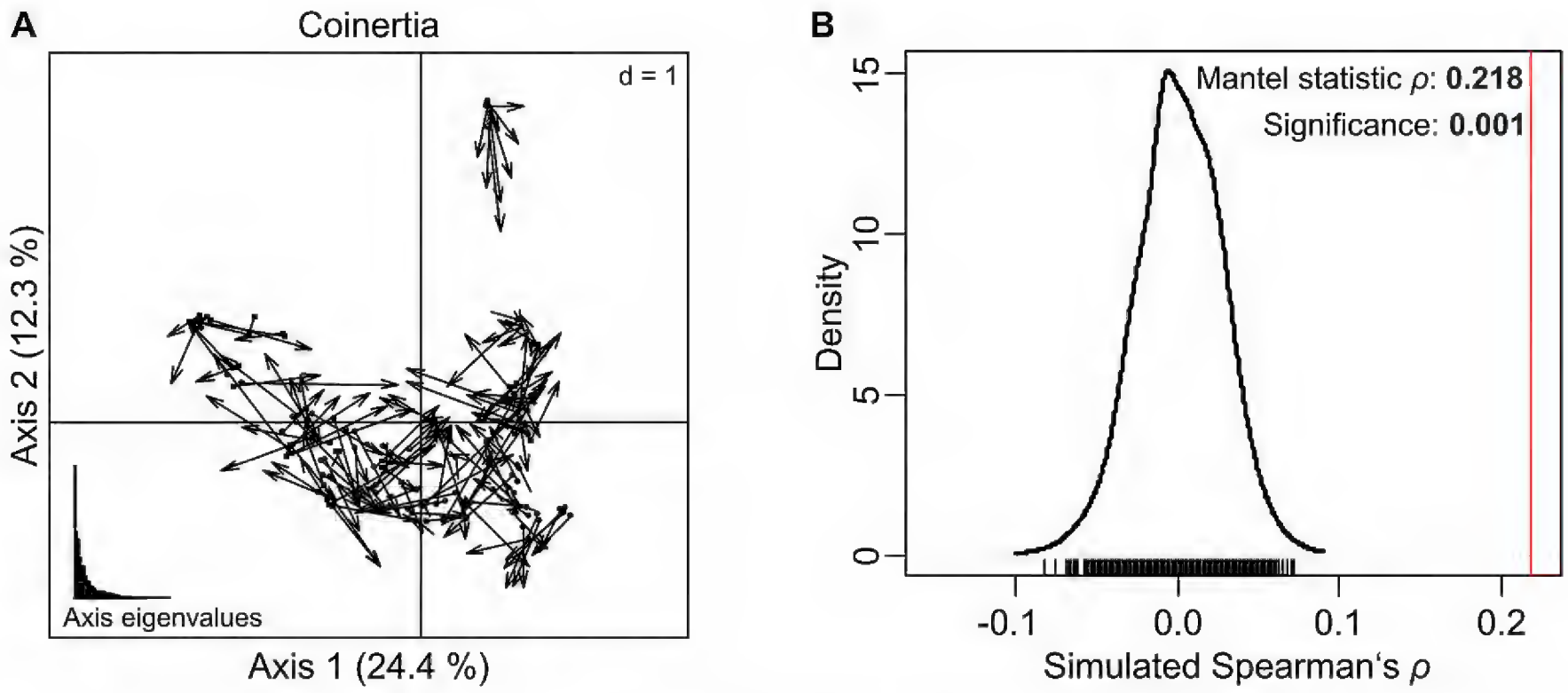

Figure 4. Differences in sample constellation between bulk and sediment metabarcoding datasets. **A.** Arrows in the coinertia diagram connect the corresponding pairs of bulk vs. sediment samples in a common ordination plane; **B.** Mantel test (right) compares the observed level of within-pair correlation (red line) with a set of random permutations (black ticks and density curve).

A second and more significant line of confirmation comes from the hierarchical clustering and ordination of detected communities. These results naturally reinvigorate the traditional discussion of how Tatra Mountain lakes should be classified. While a more comprehensive dataset from all seasons and lakes in our sampling campaign would enhance this, our current findings already correspond to earlier studies in terms of gradient patterns. Two distinct lake groups emerged from the analyses, corresponding to differences in altitude, calcium concentration, alkalinity, and related variables (Fig. 3). Additionally, key nutrients and organic compounds (TP, NO_3-N, DOC, TON) explained the segregation of two lakes from the main group of oligotrophic lakes. As expected, lakes in the high-altitude group showed lower taxonomic richness than those in the low-altitude group.

The observed grouping aligns with the two altitudinal categories of Tatra Mountain lakes proposed by Krno et al. (2006). Earlier classifications, such as those by Hrabě (1939, 1942), recognized (sub)alpine lake groups based on clitellate and chironomid fauna. However, those classifications were based solely on profundal sediment samples, where the mutual exclusion of *Procladius* and *Stylodrilus* in alpine-meadow versus dwarf-pine zones was interpreted as an indicator of varying lakebed oxygenation. Unsurprisingly, these classes did not match patterns in our littoral samples, nor those in Bitušík et al. (2006). In our indicator species analysis, a single *Procladius* sp. OTU showed low fidelity across both groups (~0.2), but with strong specificity to alpine lakes (0.85, BOLD:ABX4068). Other *Procladius* BINs appeared more associated with the forest zone, such as *P. crassinervis* (BOLD:AEE7861) and *P. pruinosus* (BOLD:ACY8325). *Stylodrilus* OTUs were detected in all lakes except Nižné Kozie and two forest lakes from our fixative-DNA dataset (Vargovčík et al. 2024).

The classification by Krno et al. (2006) was congruent for 8 of the 10 lakes evaluated in both studies. In the early 2000s, littoral fauna-based classifications were shaped by the period of acidification (Krno 1991; Bitušík et al. 2006), and these lakes have since been recovering or transforming under

changing environmental conditions (Stuchlík et al. 2017; Kopáček et al. 2019). That earlier distinction into acidified versus non-acidified lakes (Krno et al. 2006) agrees with the subdivision within our high-altitude group in Fig. 3. Nonacidified lakes (Litvorové and Nižné Terianske) cluster separately from the previously acidified ones—although this distinction was not well supported by hierarchical clustering of metabarcoding data.

Two lakes—Pusté and Vyšné Zbojnícke—appear to have shifted from alpine to subalpine classification in our study (Fig. 3). This is consistent with their intermediate chemical profiles (Suppl. material 3: fig. S5). Explaining the shift solely by suggesting post-acidification recovery (Stuchlík et al. 2017) would contradict their previous categorization as nonacidified (Krno et al. 2006). However, taxon list for these two lakes was now enriched by multiple genera assessed in earlier studies (*Cernosvitoviella*, *Mesenchytraeus*, *Tubifex*, *Pisidium*, *Hydroporus*, *Acrophylax*, *Rheocricotopus*, *Zavrelimyia*), and metabarcoding could also resolve multiple species within the previously reported Hydracarina, Micropsectra spp. and other unreported groups (Bitušík 2004; Krno et al. 2006; Novikmec et al. 2015). The presence of subalpine-associated taxa, and the absence of alpine-indicators, may explain the apparent reclassification.

In contrast, the neighboring Ľadové Zbojnícke lake has consistently remained in the alpine group—likely due to its greater depth and smaller catchment area, which limit warming and nutrient input (Kopáček et al. 2024). Consistently, low-altitude indicator species like *Agabus bipustulatus* and *Micropsectra notescens* were absent from this lake but found elsewhere in the valley (Bitušík 2004; Krno et al. 2006). Orographic barriers and limited hydrological connectivity may also contribute to this lake's distinctiveness.

The altitudinal reclassification of Pusté and Vyšné Zbojnícke likely reflects a combination of methodological improvements, environmental change, and ecological connectivity (Kopáček et al. 2015; Bitušík et al. 2017; Kopáček et al. 2019). Additionally, the apparent upward expansion of *Macropelopia nebulosa* (BOLD:AAX3566) was notable—its OTUs were found in all lakes (except Ľadové Zbojnícke and Štrbské) in 2022, whereas it was previously absent from most high-altitude lakes.

Species previously proposed as indicator taxa generally matched our observations (Krno et al. 2006; Hamerlík and Bitušík 2009). For alpine lakes, *Pseudodiamesa nivosa*, *Micropsectra radialis*, and *Capnia vidua* were confirmed. Their occasional presence in low-altitude lakes supports earlier reports of low-frequency occurrences outside their main zones. For subalpine lakes, *Micropsectra* spp., *Eukiefferiella minor*, *Prodiamesa olivacea*, *Nemurella pictetii*, and *Agabus bipustulatus* were upheld as indicators. However, incomplete species records and narrow distributions limited confirmation of other candidate indicators (e.g., *Pedicia rivosa*, *Leuctra rosinae*, etc.). Four previously proposed indicator species were not detected at all—despite sampling up to five lakes where they had been found before. For two (*Cernosvitoviella tatrensis*, *Zalutschia tatrica*), poor representation in BOLD may explain their absence. Enhancing barcode libraries (Weigand et al. 2019; Csabai et al. 2023; Meier et al. 2024) and recent upgrades in BOLD enabling private BIN matching (Ratnasingham and Hebert 2007; Buchner and Leese 2020) are expected to reduce such gaps.

Although indicator species have never been absolutely restricted to specific altitudinal categories (Krno et al. 2006; Hamerlík and Bitušík 2009), their

distributional shifts—especially in Pusté and Vyšné Zbojnícke—support our observations of ecological transition. Species like *Zavrelimyia melanura*, *Diura bicaudata*, *Nemurella pictetii*, and *Eukiefferiella minor* reflect this shift. Signs of post-acidification recovery are also seen in the presence of *Micropsectra* sp. and *Z. melanura* in Starolesnianske Lake, and even acidophobic species like *D. bicaudata* and *Ameletus inopinatus* in high-altitude lakes such as Vyšné Wahlenbergovo, Ľadové Zbojnícke, and Capie.

No species with multiple OTUs showed clear intraspecific structuring along environmental gradients (Suppl. material 1: table S3), but this may result from clustering parameters optimized for species-level resolution. For deeper population structure in glacial lakes, other genetic markers or ASVs would be more suitable (Andújar et al. 2021). Nonetheless, several species displayed unexpected levels of genetic divergence—11 species split into more than 25 BINs—highlighting both the limits of current thresholds and the power of metabarcoding for detecting cryptic diversity (Cordier et al. 2018).

The performance of sediment metabarcoding paralleled patterns seen in water eDNA studies. Highly degenerate primers often amplify nontarget DNA, resulting in bacterial, algal, or fungal dominance (Leese et al. 2021). Where a similar risk was observed for fixative (Zizka et al. 2018), our analogous samples from the Tatra lakes performed adequately (Vargovčík et al. 2024). Rather than using insect-specific primers, we employed universal primers to capture a broader range of animal DNA. While this approach worked well for preliminary tests, its effectiveness decreased in the full sediment dataset.

Still, primer degeneracy enabled the unique detection of phyla such as Gastrotricha, as well as specific taxa like *Attheyella wierzejskii* (harpacticoid) and *Cypria* (ostracod). Bulk processing—whether fixative DNA or size-sorted samples—may provide even broader animal coverage and enhance scalability (Pereira-da-Conceicoa et al. 2021; Ascenzi et al. 2024; Vargovčík et al. 2024).

A striking pattern emerged when comparing all invertebrate OTUs: those found in both sample types (113 OTUs) were almost exclusively aquatic (95.6%), whereas bulk-specific OTUs were 54.7% aquatic and sediment-specific OTUs were only 42.2% aquatic. However, sediment results were extraordinary even in their non-aquatic invertebrate OTUs, whose classification as aquatic or terrestrial was hindered by insufficient taxonomic assignment in 77 cases (mostly just order Coleoptera), contrasting with only 6 such cases from bulk. Thus, improved primer specificity, sampling strategy, and target selection (Pearman et al. 2020; Leese et al. 2021) could make sediment data more informative for biodiversity assessments.

Conclusion

This study demonstrates the effectiveness of DNA metabarcoding in assessing aquatic invertebrate diversity across an altitudinal gradient in the Tatra Mountains. The method is highly recommended for exploring the fauna of lakes and their catchments and for tracking biotic responses to environmental changes. Bulk samples provided more comprehensive taxonomic coverage than sediment samples, confirming their value for biodiversity monitoring. The distribution of identified OTUs aligned with environmental gradients, with altitude, nutrient levels, and water chemistry shaping macroinvertebrate communities. Two lake groups emerged clearly, although some lakes exhibited intermediate

physico-chemical traits. Several known indicator species were confirmed, while others were not detected—likely due to gaps in reference databases. Shifts in altitudinal classifications of certain lakes reflect ecological change driven by climate, acidification recovery, and hydrological connectivity. Future work should expand coverage across the mountain range, continue enriching barcode libraries, and analyze seasonal and broader taxonomic datasets to improve lake classification and understand macroinvertebrate responses to change.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Use of AI

No use of AI was reported.

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Data availability

The demultiplexed and primer-trimmed FASTQ files from the MiSeq analysis have been uploaded to the NCBI Sequence Read Archive (Project ID PRJNA1182547).

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Supplementary material 1

Additional tables

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Data type: pdf

Explanation note: **table S1.** Morphological and catchment parameters of the studied lakes. Lake codes correspond with Table 1. **table S2.** Lake water composition in the recent years. **table S3.** Indicators recognized from the bulk metabarcoding dataset.

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Supplementary material 2

Supplementary methods

Authors: Ondrej Vargovčík

Data type: pdf

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Supplementary material 3

Additional figures

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Data type: pdf

Explanation note: **fig. S1.** Rarefaction curves based on the metabarcoding dataset. **fig. S2.** Aquatic OTU counts and read proportions for invertebrate orders detected with sediment metabarcoding. **fig. S3.** Cluster dendrogram for the final bulk metabarcoding dataset. **fig. S4.** Values of Pearson's correlaton coefficient (PCC) calculated from the matrix of environmental parameters of the 17 sampled lakes. **fig. S5.** Principal coordinate analysis from individual measurements of physicochemical lake parameters. **fig. S6.** Distance-based redundancy analysis for the curated sediment metabarcoding dataset.

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